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THE MICRO-ORGANISM OF FAULTY RUM

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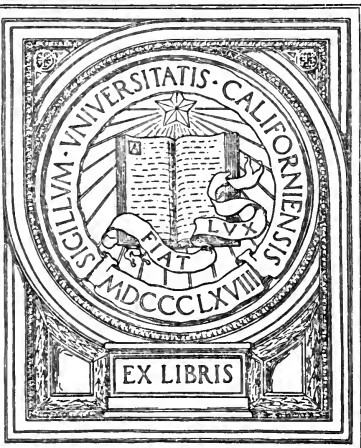

THE

MICRO-ORGANISM

OF

FAULTY RUM

BY

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AND

LILIAN J. VELEY (*NÉE* GOULD)

'Melius autem est naturam secare, quam abstrahere.'—BACON.

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HIS ADVICE,

HIS SCIENTIFIC INVESTIGATIONS,

AND

HIS PRACTICAL STUDIES IN FERMENTATION.

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PREFACE

In this monograph it is intended to give an account of a micro-organism which, if the views herein brought forward are correct, has caused damage to the extent of many thousands of pounds, but yet has hitherto escaped observation, mainly because it was supposed upon *a priori* grounds that no organism whatever could live in liquids of such alcoholic strength. No person previously looked for any organism, or even suspected that masses observed under the microscope could be anything but lifeless matter; it was therefore left for the authors to make the discovery of life in strong spirit.

As certain doubts were expressed upon the matter, on the one hand by those with experience who had propounded rival theories, and on the other by persons either without practical qualifications to judge of bacteriological questions in connexion with fermentation industries or personal inquiry into our results, we consulted Professor Emil. Chr. Hansen, of the Carlsberg Laboratory, Copenhagen, before venturing to lay this monograph before the public. His examination of our materials, cultures, and preparations confirmed our results, though not our conclusions in their entirety as to the identity or position of the organism or organisms, discovered by us, in any scheme of classification. For Professor Hansen's kind assistance and courtesy, as also for much time spent by him

in helping us to elucidate the matter, we render him our most cordial thanks.

Further our thanks are due to Dr. J. Ritchie, Lecturer of Pathology in this University, for advice and assistance; to Mr. G. J. Burch for conducting a number of optical tests, some of which he had previously invented, while others were specially devised for this investigation; to Mr. J. J. Manley, for certain optical measurements; to Dr. Gustav Mann, for skilful micro-photographs; and finally to certain firms and individuals, for providing us with a variety of material without which an investigation of this character would have been impossible.

In conclusion we cannot but trust that we may have opened out a new field of research in connexion with the micro-organisms of spirit distilleries, which have not, as those of breweries and wine industries, received much attention. In consequence of the large capital and the many interests involved, we have spared neither time, nor labour, nor expense to elucidate the problem of the faultiness of rum, and to make known its solution.

We have purposely entered somewhat fully into a description of all the experiments performed by us, whether resulting in success or failure, in order that our work may be repeated, and perhaps advanced in other laboratories, as we are well aware that the life-history of the organism herein described may continue for some time to be a matter of reasonable controversy.

V. H. VELEY,
L. J. VELEY.

OXFORD, *Nov.* 1, 1898.

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CHAPTER I

INTRODUCTORY

In the past few years the importations of rum from the colony of British Guiana, and to a less extent from certain of the West Indian islands, have not unfrequently exhibited the condition technically known as 'faultiness,' whereby a considerable reduction of value and consequently a pecuniary loss, amounting to several thousands of pounds per annum, has resulted, so that the manufacture of this spirit has hardly been remunerative. Under these circumstances the Agricultural Committee of the Royal and Commercial Society requested Mr. J. B. Harrison, the Government Analyst of British Guiana, to inquire into this matter, who issued a report dated Georgetown, February 16, 1897, from which the following extract is made by way of a preliminary explanation :—

'Rums have been and are classed as "faulty" when on admixture with water in such proportions as to reduce the strength to 25% under proof, which in the case of our rums means approximately their dilution with an equal bulk of water, the diluted spirits, either at once or after standing for some hours, become cloudy, either depositing on longer standing a more or less copious precipitate or showing the presence, in greater or less abundance, of floating flocculencies. This so-called test appears to be made at times with cold water, at times with boiling water, but in both cases the rum is left to stand for several hours before a final decision as to its quality is arrived at.

'Very many samples of so-called "faulty" rum have been, from time to time, sent back to this colony, and have here

been retested with ordinary rain-water or with distilled water. As a rule almost all of these samples have remained perfectly clear, and the reason for their having been classed as "faulty" has appeared to be a mystery.

'On a few occasions rums have been found to become cloudy upon dilution with pure water, and the causes of this have been proved in some cases to be the presence of a caramel in the colouring matter, easily soluble in alcohol of a strength of 40% over proof, but insoluble or with difficulty soluble in alcohol of 25% under proof, whilst in other cases the cloudiness has been traced to empyreumatic products, derived from gas liquor, present in the sulphate of ammonia which had been added to the wash to accelerate the fermentation.

'Both these causes are easily overcome.'

Without at present entering fully into the various points raised in the report, it will suffice to state that Mr. Harrison ascribes two further causes for faultiness, namely, *firstly*, the precipitation of substances contained in the rum by the lime and magnesia salts present in the Liverpool well-water (from which port the complaints have almost all been derived), as also the precipitation of part of the calcium sulphate and carbonate present in the well-water by the alcohol; and *secondly*, the extraction of resinous and other matters from the staves of the casks which produce 'flocculencies' and precipitates when diluted with water. The second point will be fully discussed in a subsequent chapter; as regards the first point, Mr. Harrison does not make clear the nature of the substances contained in the rum which are precipitated by the saline constituents of the Liverpool water. As the conclusion of the whole matter Mr. Harrison writes as follows: 'At present I am unable to discern any additional line of laboratory investigation likely to be of service in this matter, and must now leave it in the hands of the planters,' and 'If these precautions' (namely, those proposed) 'do not prove successful on the large scale, it will be necessary to carry on this investigation on other lines.'

It would appear from information received that other advice was subsequently taken, but as the report was of a private nature, we do not feel ourselves at liberty to make

any quotations therefrom. Within two months after the publication of Mr. Harrison's report our attention was called to the matter as a problem still unsolved. From the information which we had received in the course of private correspondence, we came to the conclusion that the difficulty was possibly not of a chemical nature.

Notwithstanding the apparent improbability that any micro-organism could survive in strong spirit, hitherto considered to be one of the best materials for preserving anatomical specimens, the phenomena described to us as attending the production of the turbidity led us to infer the possible presence of some bacteria, and consequently our examination of faulty samples sent to us was directed to this point. Our investigations have shown that neither the cloudiness nor the 'flocculent precipitates' are due to any particles of a chemical nature, whether suspended or deposited, but to the presence of a micro-organism which we believe to be hitherto undescribed.

We were informed that the first samples of coloured rum received by us direct from a bonded warehouse had been assessed by the Excise as at 42% over proof, equivalent to 74·63% per weight of alcohol. Our determinations, using standardized instruments, gave a value of 42·23% over proof, or 74·88% per weight of alcohol. A sample of white rum was found to have a value of 38% over proof, equivalent to 72·09% per weight of alcohol. In all cases we observed a small quantity of sediment at the bottom of the bottles. Mr. Harrison in his report alludes to such sediments in the following terms:—

'The diluted "faulty" rums were next examined, and the sediments separated.

'Microscopically these sediments were found to consist principally of amorphous matters mixed with occasional depolarizing crystals, probably of sulphate and of carbonate of calcium.

'By analytical examination the major portion of the precipitate was found to be organic, whilst the inorganic matters consisted of, after ignition, calcium sulphate and the carbonates of calcium and magnesium. The bulks of these sediments were far greater than if they had consisted only

of the calcium and some of the magnesium salts present in the water used for dilution.'

Mr. Harrison in another passage expresses himself as follows:—

'It was found that the amorphous organic matters precipitated by the addition of the well-water to the "faulty" rums were largely soluble in solutions of caustic soda or potash, or of the hydrate or carbonate of ammonia.'

Our microscopical examination of the sediments of faulty rum led us to a result wholly unforeseen by Mr. Harrison; the deposit was certainly organic, but only 'amorphous' in the chemical sense of the word. For with a magnification of 1,200 diameters this sediment was found to consist of a micro-organism of coccus form occurring more generally in pairs and groups, but also singly and in chains; the cocci were yellowish in colour, surrounded by a very thick, highly refractive colourless envelope, and appeared to be non-motile [Pl. I. Fig. 1, *a*, *b*].

It is not altogether surprising under these circumstances that Mr. Harrison could not obtain much residue 'after ignition,' and it would appear to be a somewhat strong measure to digest an organism in 'solutions of caustic soda or potash, or of the hydrate or carbonate of ammonia.'

It was obvious that we had before us a bacteriological problem of considerable interest and importance, and it appeared most desirable to ascertain, firstly, if the organism was living in the strong spirit; secondly, if it was really the cause of the faultiness; and thirdly, its origin. As regards the first point continuous microscopical examination showed that the organism, in samples of recent importation, was dividing actively, and cultures, hereafter to be described, were made from it with success. This fact alone of the existence of life in alcohol of such concentration is, we believe, quite new to science; the only record in literature appears to be the work of Bokorny [1], who showed that bacteria survived in alcohol of not more than 1%.

The second point will be fully discussed in subsequent

chapters, and the evidence therein brought forward will, it is hoped, be of a convincing nature.

As to the third point, we can only adduce such information as we have been able to obtain from the planters, trade-circles, and private sources without a personal visit to the infected colonies.

Considerable difficulty was experienced in obtaining exact data and various statistics from the planters, which would have thrown much more light upon the subject.

It appears that the faultiness of rum was first noticed about sixteen years ago, and it has been manifest intermittently, but with increasing frequency, since that date. The following abstract of returns obtained from a private source will serve to illustrate these points:—

Year.	*Estate A. Percentage of faulty rum.*	*Estate B. Percentage of faulty rum.*
1892	38·5	Nil.
1893	8·0	3·4
1894	Nil.	9·4
1895	Nil.	10·4
1896	50	90 (about).

The proprietors of one of the estates, who received from us a report upon the subject, have advised us that their crops since made have been practically sound, while those of other manufacturers have been even more faulty than before. Further comment appears superfluous.

Preliminary accounts of our investigation have been published in *Nature,* vol. lvi. p. 197 (1897); *Sugar Cane,* 1897, p. 350, and 1898, p. 381; *Natural Science,* vol. xi. p. 87, (1897); *The Argosy,* Demerara, July 3, 1897; and noticed in *Die Umschau,* Jahrgang 1, p. 542 (1897). Cultures and microscopical preparations of the organism were exhibited at a Conversazione held at the Oxford University Museum, May, 1898, by the Oxford University Junior Scientific Club.

CHAPTER II

GENERAL EXAMINATION OF SAMPLES

I. Bacteriological Examination.

With a view to elucidating the problem we obtained through various manufacturers and brokers a number of samples of crops from British Guiana and various West Indian islands, comprising importations of the years 1887 to 1897, of different brands, consignments to different ports, from the same ship in different years or different ships in the same year, diluted and undiluted, coloured and uncoloured, all certified as sound or faulty respectively by the brokers. Samples were also obtained independently through shops, without giving any information as to the purpose for which they were required. The results of the examination are given below in the form of short notes, which are collected together in a table.

Sample I, 1887. Coloured, undiluted, certified as sound, contained débris of sugarcane but no micro-organism.

Sample II, 1891. Coloured, undiluted, certified as sound. Good clean sample.

Sample III, 1893. Same as No. II.

Sample IV, 1894. Coloured, undiluted, certified as sound. Same as No. I.

Sample V, 1897. Coloured, undiluted, certified as faulty. Large colonies of micro-organism found, not only in the sediment, but throughout the liquid.

Sample VI, 1897. Coloured, undiluted, certified as faulty. Drawn from bottom of puncheon. Numerous colonies of large cocci in thriving condition in drops taken from the bottom,

identical cocci of smaller size in drops taken from the top. More colonies in one field of the microscope than in any other sample examined.

Sample VII, 1897. Coloured, undiluted, certified as faulty. A drop taken from the bottom showed numbers of cocci, both singly and in chains.

Sample VIII, 1897. Coloured, undiluted, certified as faulty. Large cocci from bottom drops, small cocci from top drops.

Sample IX, 1897. Coloured, diluted $\frac{1}{3}$ spirit, $\frac{2}{3}$ water, certified as faulty. Millions of cocci in all stages of development, also débris of sugarcane with colonies clustered upon them.

Sample X, 1897. Coloured, diluted $\frac{1}{3}$ spirit, $\frac{2}{3}$ water, certified as faulty. Similar as IX, but a few motile bacilli found.

Sample XI, 1897. Coloured, diluted $\frac{1}{3}$ spirit, $\frac{2}{3}$ water, certified as faulty. Large masses of cocci clustered upon débris of sugarcane.

Sample XII, 1897. Coloured, undiluted, certified as sound. Small colony found in one of many drops.

Sample XIII, 1897. Coloured, undiluted, certified as faulty. Drawn from bottom of puncheon. Chiefly small cocci in drops taken from the top and bottom.

Sample XIV, 1892. Coloured, undiluted, certified as faulty. Plenty of large groups of cocci.

Sample XV, 1897. Coloured, undiluted, certified as sound. Contained no trace of the organism.

Sample XVI, 1897. Coloured, undiluted, certified as faulty. Drops taken from the bottom contained enormous quantities of cocci collected in masses.

Sample XVII, 1897. Coloured, undiluted, certified as faulty. Large cocci in bottom drops, small cocci in top drops.

Sample XVIII, 1897. Uncoloured, diluted $\frac{1}{2}$ spirit, $\frac{1}{2}$ water, certified as faulty. Millions of small cocci aggregated together with a tendency to rise to the top of the liquid in the form of a scum.

Sample XIX, 1897. Uncoloured, undiluted, certified as faulty. Same organism as that present in No. XVIII and the coloured samples.

Sample XX, 1897. Uncoloured, undiluted as taken direct

from still in an infected works. Free from any trace of the organism.

Sample XXI, 1897. Coloured, undiluted, certified as faulty. In this sample, which had been subjected to some process of fining, large colonies were found at the bottom only. The cocci were of enormous size, but dead, as was proved not only by their abnormal appearance [Pl. I. Fig. 1, *c*], but by failure to obtain growths from them when transferred to favourable media.

It is clear from the following tabulation of the results that the micro-organism was present in the faulty, but absent from the sound samples, with the solitary exception of No. XII, in which the quantity present was not sufficient to cause cloudiness when examined by the broker's test of dilution.

No. of Sample.	*Year of Importation.*	*Dilution.*	*Condition.*	*Micro-organism.*
I.	1887.	Undiluted.	Sound.	Absent.
II.	1891.	,,	,,	,,
III.	1893.	,,	,,	,,
IV.	1894.	,,	,,	,,
V.	1897.	,,	Faulty.	Present.
VI.	,,	,,	,,	,,
VII.	,,	,,	,,	,,
VIII.	,,	,,	,,	,,
IX.	,,	Diluted.	,,	,,
X.	,,	,,	,,	,,
XI.	,,	,,	,,	,,
XII.	,,	Undiluted.	Sound.	Trace present.
XIII.	,,	,,	Faulty.	Present.
XIV.	1892.	,,	,,	,,
XV.	1897.	,,	Sound.	Absent.
XVI.	,,	,,	Faulty.	Present.
XVII.	,,	,,	,,	,,
XVIII.	,,	Diluted.	,,	,,
XIX.	,,	Undiluted.	,,	,,
XX.	,,	,,	Fresh from still.	Absent.
XXI.	,,	,,	Faulty.	Present.

These collected observations afford, to our minds, strong *prima facie* evidence that there is a relation between the presence of the micro-organism and the 'faultiness.' As the fact that the presence of the micro-organism always accom-

panies the turbidity or faultiness is of itself no proof that the former is the sole cause of the latter, the scientific method of Concomitant Variations was applied, namely, experiments were made with a view to converting samples of both undiluted and diluted faulty rum into sound samples by removing the organism, and conversely of converting sound into faulty rum by its addition.

The first experiment consisted in passing a diluted faulty sample through a sterilized Müncke's filter at a reduced pressure of about 650 m.m.; though the turbidity disappeared for the most part after the first filtration the process was repeated, the filter being meanwhile brushed and cleansed inside and outside and again sterilized. After the second filtration the liquid was not only quite bright and clear, but completely free from the micro-organism; optical tests were applied as described in Chapter IV. The same experiment was repeated with an undiluted faulty sample with the same result, and further the twice filtered liquid when diluted in the proportion of $\frac{1}{3}$ spirit and $\frac{2}{3}$ water still remained clear, as evidenced by the optical tests.

Conversely, a pint bottle of undiluted faulty rum was passed twice through another and new sterilized Müncke's filter; the sediment left thereupon was transferred to an equal bulk of sound rum contained in a similar bottle. As, however, there was a considerable slimy deposit of the organism left on the walls of the first bottle, the latter was cut in half, the deposit detached by a sterilized scoop, the whole completely cleared out and added to the sound rum mentioned above. The liquid thus obtained had all the appearance of faulty rum, and subsequent dilution in the usual proportions rendered it more apparent, and by the optical tests the characteristic phenomena were obtained.

The necessity for more than one filtration, and for repeated cleansing and sterilizing of the apparatus, would be obvious to experienced bacteriological investigators; it does not appear that Messrs. Harrison and Scard (communication to *Sugar Cane*, vol. xxx. pp. 410 et seq.) performed the operation more than once in any case. The following quotation from

Professor Hansen's well-known work [2] will show that he is in accord with us upon this matter. 'In the course of these experiments, however, I perceived that the Chamberland filters would be of great service in preparing different sterile liquids for laboratory experiments. If an absolutely germ-free filtrate is required, the tubes must be sterilized at short intervals; they must not be kept in action for an indefinite period, as was formerly imagined, for the bacteria pass through the walls after a longer or shorter period[1].'

As the faultiness is dependent upon the presence of the organism in considerable numbers, as illustrated from Sample XII, it would have been useless to have inoculated sound rum with one cell, or even a few colonies, especially as it was found that its power of reproduction by division was either lost or much impaired after prolonged life in spirit. Hence the bulk for bulk experiment above described appeared the most practicable.

Another method would have been to have cultivated the organism on a large scale in sterilized solutions of caramel or brown sugar, filtered it off, and transferred the colonies in such bulk as that present in faulty rum to an equal bulk of sound rum.

At an early part of the inquiry we inoculated samples of sound rum with a few colonies of the micro-organism, but since its power of multiplication by division soon becomes latent in the spirit, the results, as was to be expected, proved negative; if, however, the organism was transferred, even after a length of time, to a suitable medium, growth took place in the course of a few days, thus proving that the organism was not dead, but merely in a resting stage. It appears that Messrs. Harrison, Scard, and Daniels (loc. cit.) performed similar experiments with the same negative result, and arrived at the hasty conclusion that the organism was dead, but there is no evidence in the paper above cited that

[1] Cf. Migula, *Separatabdruck Jahresber. Ver. Natur*, Mannheim, 1894. 'Wollen wir nun die Wirksamkeit eines Filters prüfen, so bleibt uns dazu nur das Mittel, die Zahl der in unfiltrierten und die der in filtrierten Wasser vorkommenden Bakterien festzustellen und mit einander zu vergleichen.'

these writers, either in this case or in any other, attempted to grow the organism in a suitable medium, such as a saccharine solution; it cannot be supposed that the organism is existing in favourable conditions in rum of such alcoholic strength. No person of experience in horticulture would sow seeds on a cinder-path and expect to obtain a good flower-bed. The production of disease in animals by inoculation with a single cell of a pathogenic organism could not be cited as a parallel case, as here the nidus or medium is suitable for growth and reproduction. It will be obvious that the effects are to be measured in the case of faulty rum by the relative proportion of the micro-organism, and not by the absolute quantity of the liquid, and therefore the addition of '10%,' or even more, of faulty to sound rum would not necessarily render the latter faulty, as of course in an experiment of this kind, described by Mr. Harrison, he was not only increasing the quantity of liquid by admixture, but actually decreasing the relative proportion of the organism.

Experiments of the kind made by Messrs. Harrison and Scard are not such as to satisfy the elementary requirements of bacteriological investigation, since there is no mention of any sterilization of vessels and materials used, and one set of experiments was conducted with 'stoppered' bottles; hence the most obvious precautions against error were neglected. This is the more striking in view of the following passage in Messrs. Harrison and Scard's communication, which we cannot but regard as naïve in the extreme:—'Any person acquainted with the interiors of distilleries in the tropics would not be surprised by the presence of the remains of different organisms in the sediments of the casks, although he might be surprised if they were entirely absent.' We are not aware that any 'surprise' has been expressed as to the existence of micro-organisms in tropical distilleries; it would, however, be a matter of great surprise if, after the investigations of Pasteur and Hansen on the micro-organisms of fermentation, no steps were taken for their removal. The increased difficulty of obtaining a germ-free product in the tropics does not relieve the manufacturers from the necessity,

if they do not wish to be left behind in the trade struggle for existence.

As one of us is a director and part proprietor of a large and successful brewery, conducted on scientific principles, and the other is not without practical experience in the bacteriology of fermentation, we feel ourselves to some extent entitled to express our opinion on this point.

We think that Mr. Harrison is sanguine in expecting that 'the removal of the microbe by death' should cure the rum of its faultiness, especially as he finds its 'apparent remains' in the sediment; nor can we enter into Mr. Harrison's frame of mind, when in one passage he definitely states (p. 412) that he found the organism described by us, while in another (p. 414) he refers to it as the 'alleged bacterium.'

Examination of Caramels used for colouring.

Two samples of caramel were obtained, one direct from and recently manufactured in an infected works, the other made in England by a well-known London firm. The former was very strongly acid, and a small quantity partially dissolved in sterilized distilled water and filtered through muslin showed innumerable swarms of cocci, identical with those in faulty rum, as well as an actively motile rod form. The latter was neutral and contained only débris of vegetable fibre, and when used for certain colouring experiments of redistilled rum, gave perfectly bright and clear spirit.

One source of infection is therefore the caramel, which would form a very suitable nidus for the micro-organism, especially if it is kept, while cooling, in open vessels; though it is clear that this is not the only source of infection, since the micro-organism is absent, as we should expect, from the rum taken fresh from the still (Sample No. XX), but present in much of the uncoloured rum, which must have been kept in infected vats and puncheons.

Examination of Liverpool water used for dilution.

Three samples of Liverpool water as used by the brokers in their test were sent to us; no organism in the least resembling

that of faulty rum was found, the few bacteria present were common water forms such as might occur in any sample of tap-water.

II. Chemical Examination.

On fractional distillation of coloured undiluted spirit it was found that all the volatile portion came over below 100·5° C., the first and greater portion distilled at 78·5°–79°, the boiling point of ethyl alcohol, the second portion, from 80° to 98°, was milky in appearance and probably consisted of compound ethers with water, and the third portion of 99°–100·5° of water only. This suffices to prove the absence of any substance of the nature of a terpene which might be extracted from the wood by the spirit, and precipitated therefrom on dilution.

In order to determine the materials other than alcohol, traces of ethereal salts, and water, determinations were made of the solid residue at 100°, and the residue on ignition in the case of two samples, one coloured and the other white; in the former case 86·06 grams yielded on evaporation over a water-bath ·938 gram solid residue, which on ignition gave a residue of ·0635 gram; in the latter case 86·7 grams gave ·0145 gram of residue dried at 100, and an inappreciable residue on ignition. The food material in the first case consisted, therefore, of 1·01% carbohydrates, and ·074% inorganic salts, reckoned as anhydrous, and in the latter case of ·016% carbohydrates only.

It was thought, at the outset, that there might be some relation between the faultiness and the proportion of free acid, but analyses of various samples showed that sound spirit might contain a greater quantity of free acid than 'faulty' spirit. It is, therefore, clear that the free acid produced in the original fermentation and volatilizing over on distillation might be in excess of that produced by the subsequent decomposition of the sugar contained in the added colouring matter by the micro-organism.

But for the better purpose of comparison the amount of acid per unit weight was determined in the same faulty spirit, (1) undiluted and (2) diluted, some months previous to

examination in the proportion mentioned above. The following determinations are selected to illustrate this point; the values are given, according to the usual convention, in terms of acetic acid, and though it is most probable that this is not the only acid present, yet the conclusion deducible is not materially affected.

Sample.		*Proportion of acid to 100 grams spirit.*	
A	Diluted	·1216 gram	Coloured Samples.
	Undiluted	·0961 „	
B	Diluted	·1176 „	
	Undiluted	·0849 „	
C	Diluted	·1418 „	
	Undiluted	·0946 „	

In all cases the amount of free acid in the diluted was greater than that in the undiluted samples, thus showing that some decomposition of the caramel or alcohol had taken place by the agency of the organism, when living in the diluted spirit.

In this connexion there is an experiment described by Messrs. Harrison and Scard (loc. cit. p. 414) in which it is stated that the sediment from faulty rum was soluble in alcohol of 40 to 50°/₀ over proof, and the solution after filtration through a Pasteur-Chamberland filter became opalescent on dilution. From the terms used it would appear that the sediment is the same as that which we find in undiluted faulty rum of 40°/₀ over proof; if this is the case, the authors have succeeded in finding a substance, bacterial or resinous, which is at once precipitated out as a sediment, in spirit of 40°/₀ over proof, but is also soluble in the same menstruum. If this experiment had not been described in print, we should hardly regard it as worthy of the serious attention of scientific persons.

III. Physical Examination.

These phenomena will be dealt with in detail in the two succeeding chapters, wherein a full discussion of Mr. Harrison's theory will be found; meanwhile we would desire to point out that in the recent paper above quoted no tests seem to

have been applied for (i) opalescence and (ii) fluorescence; this would seem to be the more necessary as the authors evidently confuse four totally distinct phenomena, namely, the two just mentioned, (iii) turbidity, and (iv) alteration of refractive index.

It may be convenient to remind those of our readers who are interested in the methods of physical science, that the three words *turbidity, opalescence,* and *fluorescence* are employed to represent three separate phenomena, each with its special significance and connotation.

We denote a liquid as *turbid* when it contains suspended particles which disturb the passage of light through it, rendering it thick without causing any alteration in the character of the light they reflect. *Turbidity* indicates the presence of particles visible at any rate under the microscope.

The term *opalescence* we have restricted entirely to that phenomenon as originally described by Tyndall, especially with reference to the blue waters of the Alpine lakes and blue sky, which owe their colour to the presence of innumerable particles so fine as to be invisible under the highest magnification. Light reflected by such particles, when passed through a Nicol's prism at right angles to the line of incidence, is invariably polarized.

Fluorescence consists, as is well-known from the investigations of Sir G. Stokes [3], essentially in the emission by the substance of a light of a different colour from that which falls upon it. Many resins have the property, such for example as a solution of catechu in alcohol, which gives a green fluorescence, and guaiacum, which gives a blue fluorescence. But when such solutions of resins are mixed with water the *fluorescence disappears, but opalescence appears.* It will be our purpose to demonstrate that the faultiness of rum is properly described primarily as a *turbidity,* but also as an *increased fluorescence,* due to the fact that the organism extracts and assimilates that particular ingredient of the caramel, which is fluorescent; *dilution of rum with water in no case causes an opalescence.*

CHAPTER III

THE 'RESIN' THEORY

In the report of Mr. Harrison alluded to above the following passage occurs:—

'When the spirits were kept in contact with the split-up staves [of casks used by manufacturers] for two or three weeks, the alcohol took up from them considerable quantities of resinous and other matters, so as to produce flocculencies and precipitates when mixed with distilled water or with rain-water.

'Similar experiments were made with split-up staves, using coloured rum which had successfully stood the test with the Liverpool well-water, and it was found that after being in contact with the pieces of staves for a few days, the coloured rum acted in a manner not distinguishable from that in which the faulty rum acted when mixed at 50° F. with the Liverpool well-water.

'From this it appears that the deleterious substances producing cloudiness when rum is mixed with Liverpool well-water probably are derived from the staves of the cask by the solvent action of the alcohol upon certain of the constituents of the wood.'

Mr. Harrison does not bring forward any direct evidence that the matters extracted from the staves of the casks were of a resinous nature; it does not even appear that he applied any of the well-known physical or chemical tests for resins, neither does he offer any proof that the 'flocculencies' and 'precipitates,' which he obtained after extraction of the wood with the spirits and subsequent dilution, were identical in character with the 'floating flocculencies' which he found in faulty rum.

Further, it appears from information obtained from the manufacturers that the faultiness, though known for at least fifteen years, has been of an intermittent character, whereas the wood used for puncheons, vats, &c., has been constant; again, at certain times, some Demerara manufactories were attacked and not others, yet there is good ground for believing that all the firms were supplied with the same timber.

Notwithstanding the weakness of the evidence brought forward to support the view that the faultiness is due to resinous matter extracted from the wood, it seemed desirable to ascertain if the presence of such matters could be detected by microscopical, chemical, or physical tests, and as it will be shown in the sequel that negative results were obtained, the method of difference was applied, namely, that certain resins were added both to distilled and coloured spirit, and the effects produced compared with the faultiness of rum.

Microscopical Tests.

In the course of the investigation many hundred drops were examined under the microscope; these included all the deposits of every character, both from diluted and undiluted samples. But apart from the micro-organism, only débris of definite vegetable structure, probably sugarcane, and in a few cases crystals resembling calcium sulphate in appearance, were found; the magnification used varied from 600 to 1000 diameters. No amorphous substance, which might be of the nature of a chemical precipitate, was ever detected.

The above evidence, though pointing to the improbability of Mr. Harrison's views, yet cannot be accepted as definite, as Picton and Linder [4] have observed that certain so-called pseudo-solutions of mercury and other metallic sulphides showed a clear field with a magnification of 600 diameters, and even with 1000 diameters the resolution of such solutions into its constituent particles was not always very apparent. These writers further showed that though such fine particles were not always retained by a dialyser of vegetable membrane, yet they in no case passed through a porous cell; this point will be alluded to subsequently.

Chemical Tests.

About three-quarters of a pint of undiluted rum, classed as faulty, was passed through a bacterial filter and subsequently distilled to dryness in a capacious flask with a long neck; the distillate was reserved for experiments to be described. The brown residue obtained was first extracted with ether, which removed a faint trace of a yellow colouring matter, probably one of the constituents of the caramel; the solution was evaporated and tested with stannic chloride for a violet coloration (Renard's process) but with a negative result. The residue was then extracted with absolute alcohol, and the solution, after filtration, was tested to ascertain if any turbidity was produced on addition of (i) ether and (ii) ammonia, but also with negative results (Hirschsohn's methods); alcoholic solution of lead acetate precipitated, of course, the brown colouring matter of the caramel.

Lastly, a portion of the alcoholic extract and also the remainder of the residue was digested with light petroleum spirit, but no colouring matter was extracted nor any green fluorescence observed in the solution (Durand-Claye method).

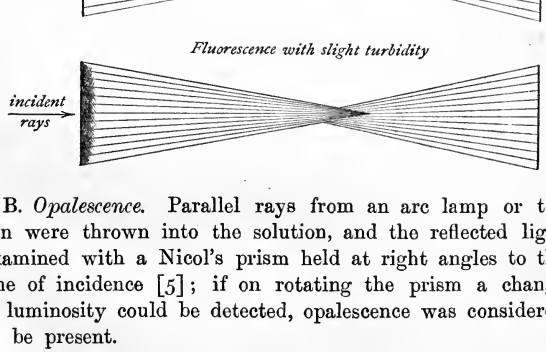
From the above it is evident that all the well-known chemical tests for resins gave negative results; further, the small amount of residue, less than 1% (*vid. supra*), obtained on evaporation of a sample of undiluted faulty spirit would equally point to the same conclusion that no appreciable quantity of a resin is present.

Physical Tests.

As it has been shown that the faultiness is not due to the presence of lifeless particles visible under the microscope, three possible causes remain: (A) *Turbidity*, due to the presence of the micro-organism; (B) *Opalescence*, due to the presence of particles so fine as to be separately invisible under the microscope, but which polarize all light reflected by them at right angles to the incident rays; and (C) *Fluorescence*. If a resin were present faulty rum would always show an

opalescence on dilution, and in any case it might be more or less fluorescent. Experiments to be described show that faulty rum is *not always* opalescent, and at any rate the opalescence is no measure of the faultiness, but all coloured rum, sound or faulty, is more or less fluorescent, and the degree of fluorescence is a measure of the faultiness. The following methods were applied for the detection of the three above-mentioned phenomena.

(A) *Turbidity* and (C) *Fluorescence.* Light from an arc lamp of about 2,000 candles, or direct sunlight, was concentrated on the surface of the liquid in an open glass vessel; it was examined both for A and C by noting the appearance at the surface of the liquid, where the light was more diffuse, and also at the focus. Simple turbidity shows most light at the focus, but fluorescence, unless very slight, is stronger at the surface than at the focus. The two diagrams given below serve to illustrate this point.



B. *Opalescence.* Parallel rays from an arc lamp or the sun were thrown into the solution, and the reflected light examined with a Nicol's prism held at right angles to the line of incidence [5]; if on rotating the prism a change of luminosity could be detected, opalescence was considered to be present.

C. *Fluorescence.* A converging beam from the arc-light or sunlight was passed through a vessel containing a solution

of ammonio-sulphate of copper of sufficient concentration to completely stop all rays of longer wave length than G', as determined by the spectroscope. The light reflected by the liquid was also examined with the same instrument.

If only the violet end was visible, and if when sunlight was used Fraunhofer lines appeared, then fluorescence was considered to be absent; but if, on the other hand, green light was reflected, showing no Fraunhofer lines, fluorescence was considered as proved.

As an example of a fluorescent resin guaiacum was selected, not of course as a substance likely to be present, but as partly soluble in alcohol, forming a solution indistinguishable in appearance from coloured rum. Two solutions were made, one containing 1% guaiacum in alcohol, and the other one part per thousand of guaiacum in redistilled and coloured rum; both were filtered through paper, and then passed through a bacterial filter in order to make the comparison with faulty rum complete; both were tested for A, B, and C before and after dilution. The results are given below.

Solution I. 1% guaiacum.

Before dilution.

A. Turbidity none; B. Opalescence none; C. A blue fluorescence.

After dilution.

A. Turbidity none; B. Beautiful opalescence; C. No fluorescence.

Solution II. One part per thousand of guaiacum in redistilled, coloured rum.

Before dilution.

A. Turbidity none; B. Opalescence none; C. A blue and green fluorescence.

After dilution.

A. Turbidity none; B. Well-marked opalescence; C. No fluorescence.

The general result of these experiments tends to show that alcoholic solutions of fluorescent resins on dilution with water lose their fluorescence entirely and become entirely opalescent.

Similar experiments were conducted with various other non-fluorescent resins in very dilute solutions, and in all

cases such solutions, after passage through a bacterial filter and subsequent dilution, became opalescent. It will be shown in the sequel that faulty rum, when dealt with in a similar manner, never becomes more opalescent.

To sum up, therefore, *firstly*, there is no evidence from microscopical, chemical, and physical tests of the presence in faulty rum of any resinous matter, and *secondly*, when a trace of a fluorescent resin is added to redistilled coloured rum, its presence can be detected both before and more markedly after dilution.

It would appear somewhat difficult of explanation that fragments of a resin could be treated with Hermann's fluid, which contains platinum tetrachloride and osmic acid, could then be washed with water and alcohol, stained with an aniline dye, and subsequently treated with xylol, but yet withal retain their original shape.

We have reason to believe that Mr. Harrison recently received large masses of the organism from works which (contrary to his belief) we, some thousands of miles away, declared to be infected; we are surprised that Mr. Harrison does not allude to this occurrence in his subsequent communication.

In conclusion, Mr. Harrison does not seem to have realized that reiteration is not proof, and that it is incumbent upon him to extract and isolate the resin from the timber used, and to determine its chemical properties and constitution, as also its physical properties, especially as regards solubility in alcohol; he must then show that the alleged resin in faulty rum is identical either with the substance as extracted from the wood, or those products of it which are dissolved out by alcohol. Otherwise he is in the position of a person who comes into a law court with a claim to the real estate of a property, but cannot produce the title-deeds.

CHAPTER IV

THE FLUORESCENCE OF COLOURED RUM AND ITS REFRACTIVE INDEX

In the introduction allusion was made to the turbidity described as apparent in the coloured spirit when diluted, for which the purchasers have claimed a rebate from the importers. It was at once observed that such samples, when viewed by transmitted light, appeared bright and clear, but opaque to a greater or less degree by reflected light, and also that this opacity could be diminished by the addition of a further quantity of spirit. As the phenomena seemed to be mysterious alike to the manufacturer and writers on the subject, but were evidently due to some optical effect, it was determined to ascertain if they could be traced to either or both of the following causes: (1) *Alteration of Refractive Index.* If the refractive index of the gelatinous envelope surrounding the micro-organism were nearly equal to that of the undiluted rum, then, on dilution with water, the refractive index of the spirit would be altered (as its density is increased) according to the well-known formula $\frac{\mu-1}{d} = C$, while that of the envelopes would remain unaltered; consequently the turbidity would become more apparent. (2) *Fluorescence*, which is known to be characteristic of many bacterial forms.

The result of the experiments tend to show that the phenomena observed is the mixed effect of both causes, as the presence of the micro-organism both alters the refractive index of diluted spirit and increases the fluorescence of the coloured spirit, whether dilute or undilute.

I. *The Alteration of Refractive Index.*

The refractive indices of four samples of rum were determined, two of which were undiluted and two diluted; of the former, one had been classed as faulty and contained a large quantity of the micro-organism, the other as sound; of the latter, one was the faulty sample mentioned above which had been diluted in the proportion of one-third spirit to two-thirds water, and the other, the same sample freed from the micro-organism by filtration through a bacterial filter.

		Values for μ_D at 19°.
Undiluted samples	(i) Faulty rum . . .	1·36545
	(ii) Sound rum	1·36597
Diluted samples	(i) Faulty rum	1·34273
	(ii) The same bacterially filtered	1·34132

Two other determinations were made of the undiluted faulty rum with a view of studying the effect of temperature, all other conditions remaining the same; the values obtained were at 21°·6 $\mu_D = 1{\cdot}36492$, and at 23°·6 $\mu_D = 1{\cdot}36491$. It will be evident from the inspection of the above figures that the refractive index of both sound and faulty rum, when undiluted, is the same, and therefore the presence of the micro-organism produces no effect; but if, as supposed above, the refractive index of the envelopes differs from that of diluted rum, then there should be a slight variation in the refractive indices of the latter when containing and when free from the micro-organism.

II. *The Fluorescence.*

The earlier experiments upon this subject were conducted in accordance with the well-known methods of Sir G. Stokes, and with one sample of diluted faulty rum; the subsequent experiments by the methods described in the previous chapter, and with both undiluted, diluted, faulty, and sound samples, as also with various samples of caramel and black sugar. The results obtained are described below.

First Series of Experiments.

Firstly, the solar spectrum was projected by means of a heliostat and prism upon a screen in a dark room; when a small flat glass cell containing the spirit was passed along the spectrum from the red to the violet, the phenomenon of fluorescence appeared most marked in the latter. Secondly, a converging beam of sunlight, when passed through a rectangular glass vessel filled with spirit, was of a beautiful green tint, but when passed through a second and similar vessel, also containing the spirit, the green tint disappeared. Thirdly, two large quartz prisms were substituted for the glass prism used in the first experiment in order to obtain a great dispersion; when the cell was placed in the violet part of the spectrum the green fluorescence was observed, and was intensified in the ultra-violet when the green fluorescence became of a violet or blue tint.

By way of comparison the experiments described above were repeated with a solution of aesculin, as one of the substances originally used in the classical investigations of Sir G. Stokes.

Second Series of Experiments (*by methods described above*, p. 19).

Sample I. Faulty Rum undiluted.

A. Turbidity, considerable.
B. Opalescence, roughly about 5% of the turbidity.
C. Fluorescence, very marked from orange to blue green.

Sample II. Rum, undiluted, which passed the brokers' test, but which contained a small quantity of the micro-organism.

A. Turbidity, negligible.
B. Opalescence, very slight.
C. Fluorescence, not so marked as in No. I.

Sample III. Another sample of faulty rum, undiluted.

A. Turbidity, considerable.
B. Opalescence, none.
C. Fluorescence, very marked.

Sample IV. Sound rum, undiluted.

A. Turbidity, none.
B. Opalescence, none.
C. Fluorescence, much less than in above samples, but evident.

Sample V. Faulty rum, diluted, not filtered.

A. Turbidity, well marked.
B. Opalescence, a trace.
C. Fluorescence, well marked.

Sample VI. No. V passed through a bacterial filter.

A. Turbidity, none.
B. Opalescence, none.
C. Fluorescence, slight.

Sample VII. Faulty rum passed through a bacterial filter.

A. Turbidity, none.
B. Not tested.
C. Fluorescence, evident.

Sample VIII. Sound rum, made faulty.

A. Turbidity, marked.
B. Opalescence, marked.
C. Fluorescence, very marked.

All the above experiments were conducted with samples which had been coloured in the usual manner with caramel; the next two were made with uncoloured samples.

Sample IX. Rum fresh from still.

A, B, C. All gave negative results.

Sample X. Rum, not coloured, but containing the organism.

A. Turbidity, considerable.
B. Opalescence, slight.
C. Fluorescence, absent.

As these experiments showed that all coloured samples, whether faulty or sound, undiluted or diluted, were more or less fluorescent, though the phenomenon was much more marked in the faulty than in the sound samples, further experiments were conducted with various samples of caramel and brown sugar, in order to determine if solutions of these samples were also fluorescent.

Sample XI. Solution of caramel, obtained from manufacturers.

A and B not tested.
C. Fluorescence, very marked.

Sample XII. Solution of canesugar.

A, B, and C gave negative results.

Sample XIII. Sample of caramel made from XII, and dissolved in water to give a solution of tint resembling undiluted rum.

A. Turbidity, none.
B. Opalescence, not tested.
C. Fluorescence, resembling sound rum.

Sample XIV. Solution of brown sugar, as used for culture experiments.

A. Turbidity, very slight.
B. Not tested.
C. Fluorescence, slight.

The above experiments show that solutions of caramel are fluorescent *per se*, a fact which, it is believed, has not as yet been observed; but, again, the phenomenon was much more marked in the solution of the caramel, which had been obtained directly from one of the works, and was full of the micro-organism. One further experiment was conducted with a gelatine culture of the micro-organism in a Petri-dish, which showed that the gelatine, originally non-fluorescent, had become fluorescent even though sown from white rum.

The general conclusions to be drawn from the results described above are as follows:—

(1) Faulty rum is turbid both before and after dilution, when tested with a sufficiently strong light.

(2) Faulty rum is never more opalescent after dilution.

(3) Sound rum is slightly, but faulty rum very fluorescent, so that it appears that the micro-organism takes up the colouring and fluorescent matters.

(4) Caramel, even when prepared from pure cane sugar, is fluorescent, and here again the presence of the micro-organism increases the phenomenon.

CHAPTER V

METHODS OF CULTURE AND EXPERIMENT

THROUGHOUT our experiments choice was made of various saccharine media for cultural experiments in preference to bouillon, agar, serum, &c., because it was desirable to imitate as nearly as possible the conditions which might obtain in the manufactories, and thus to avoid the possible production of involution-forms by the use of highly artificial media.

Solutions were made as follows in sterilized distilled water:—

I. Five per cent. and ten per cent. solutions of very coarse black sugar.

II. Five per cent. solution of cane sugar, with a trace of microcosmic salt.

III. Five per cent. solution of milk sugar, with ·5% microcosmic salt.

IV. Five per cent. solution of grape sugar, with ·5% microcosmic salt.

V. Five per cent. solution of cane sugar, with 1 % calcium chloride.

VI. Five per cent. solution of cane sugar, with 1% calcium chloride and a trace of microcosmic salt.

VII. Five per cent. solution of milk sugar, with 1% calcium chloride and a trace of microcosmic salt.

VIII. Five per cent. solution of milk sugar only.

IX. Five per cent. solution of cane sugar with weighed quantities of calcium carbonate.

X. One per cent. cane sugar, with $\frac{1}{10}$% each of calcium nitrate and gelatine.

XI. One per cent. milk sugar, with $\frac{1}{10}$% each of calcium nitrate and peptone.

XII. One per cent. starch disseminated in water.

XIII. Five per cent. cane sugar only.

XIV. Five per cent. milk sugar, with $\frac{1}{10}$% peptone.

XV. English caramel.

Cultures were also made in gelatine as follows:—

I. Deep (not stab) cultures in test tubes (*a*) with, and (*b*) without sugar.

II. On microscope slides; (*a*) with, and (*b*) without cover-glasses.

III. Shake-cultures in Petri's capsules, without sugar.

Gypsum blocks were also soaked with certain of the solutions mentioned above and sown.

Materials for Inoculation.

In the course of the investigations sowings were made from:—

I. Undiluted, coloured rum of six different brands as received from the manfacturers and brokers.

II. Ditto, uncoloured rum.

III. Previous cultures, namely, after a growth had been obtained in solutions, &c., when sown from I and II.

Methods of Sterilization and Inoculation.

Throughout our experiments all liquid media were sterilized by long boiling and subsequent discontinuous heating for two days in a steam sterilizer, but subsequently experiments were repeated with these liquids bacterially filtered (under the conditions laid down) before inoculation.

The gelatine used was obtained ready sterilized, through the kindness of Dr. James Ritchie, from the Pathological Laboratory at the University Museum, Oxford. Gypsum blocks were sterilized by passing through the flame of a Bunsen

burner. Flasks and all utensils used were first steeped in a solution of mercury perchloride (1 : 1000), and subsequently washed, boiled, and discontinuously steam sterilized with their cotton-wool stoppings. Our blank controls showed these methods to be effectual, but, in the case of cultures direct from rum, the small quantity of strong alcohol which was of necessity introduced with the inoculation proved antiseptic for other organisms.

As regards liquid media, ninety-six cultures were made in Freudenreich-Hansen flasks [see Pl. IV. Fig. XV] of 20 c.c. capacity. Inoculations were made with pipettes of 1 c.c. capacity, divided into hundredths, which were heated in a flame immediately before use, and kept in a $\frac{1}{1000}$ solution of mercury perchloride when not required; other inoculations were made with a sterilized platinum loop. Various quantities from ·1 cc. the minimum, to ·6 c.c. the maximum, of the samples of rum were introduced at a sowing, or a loopful of the cocci from a previous culture. In all cases the flasks to be inoculated were held in the hot air above the flame of a Bunsen burner, a method which, failing a germ-free chamber, is the most practicable and efficient; two operators worked simultaneously, and the processes of opening the flasks, inoculating, and closing them were performed as quickly as possible.

After having once obtained a growth, all cultures were made in sets of threes, viz. (1) from rum direct, (2) from previous cultures, (3) a blank sample as control. These were incubated (*a*) in a Hearson's incubator at a constant temperature of 35° C., in the dark, (*b*) in the same exposed to light, and (*c*) under glass in direct sunlight in a south window, at a varying temperature.

In the case of gelatine cultures the test tubes were inoculated according to Hansen's method, by sowing a measured quantity of the cocci-containing rum on the solid surface; the gelatine liquefied at first, so that the cocci became suspended in it, but soon solidified again.

Methods for Single-Cell Cultures.

With a view of studying the development of individual cells the hanging drop method in a moist chamber was applied, but after repeated trials was not found to be practicable.

The coccus, when placed in a hanging drop of the usual dimensions, remained perfectly quiescent, and did not even divide within reasonable limits of time. Other experiments showed that this was probably owing to the fact that a very considerable bulk of the nutrient medium was essential to development; the attempts, therefore, were repeated, using large moist chambers completely filled with liquid, but in these the cocci, as soon as they had become large and heavy and had begun to divide, invariably dropped off the cover-glass, and completed the cycle of development at the bottom of the liquid out of reach of observations by high powers.

Many experiments were then made with chambers specially constructed so that both the top and bottom consisted of cover-glasses, in order that the chamber could be reversed when the falling-off took place; it was hoped that some at least of the cells might attach themselves to either glass. These too proved unsuccessful, as also subsequent experiments, in which films of the cocci were dried on the cover-glass, but the nutrient liquid always soaked them off again with the same result as before.

The original method of the hanging drop, stiffened by the use of a variety of substances, such as gelatine, soluble glass, &c., was repeated, but also with unsatisfactory results.

The isolation of cells by the dilution method was not available where it was required to keep the individual cells under direct observation, because the cell could not be transferred from a large body of the liquid to the stage of the microscope until its development had already proceeded so far that the speck of growth became visible to the naked eye. It appeared, therefore, that the simplest method was the isolation of cells on a thin layer of a solid medium.

For this purpose microscope slides and large cover-glasses were covered with the thinnest possible layers of warm liquid

gelatine; when this had just solidified, a small quantity of the rum containing the organism was poured on the surface from a pipette, and immediately washed off again with sterilized distilled water.

Some cocci remained sticking to the gelatine, and in this way we obtained cells sufficiently isolated for microscopical observation of the development. Sowings were also made in Petri's capsules with the same view, but in this case inoculation was made while the gelatine was still in a liquid state, and isolation secured by shaking. As, however, our investigations indicated that on solid media certain stages in the normal cycle of development could be rendered latent or suppressed entirely, we devised a method, which may be considered as a modification of Koch's, for isolating cells on solid media, and subsequently transferring them to liquids for the purpose of growth. The thinnest possible layer of liquid gelatine was spread over a slide, the cocci-containing rum was poured on and washed off in the manner described above. The slide was then searched under the microscope for well-isolated cells, and one selected which appeared in every way normal, and which, so far as determinable by the highest powers, had no impurity either adhering to or immediately adjoining it. The position of the selected cell was then marked by drawing a square on the gelatine with the point of a fine sterilized knife, while the slide was still in position. The slide was now removed, and the size of the square reduced by four more transverse cuts made just within the first. The single cell was then searched for and found again under the microscope, to ensure that no injury had been done in the process of cutting round it, and the size of the surrounding square was again reduced in the same manner.

This process of alternately cutting squares and finding the enclosed cell under the microscope was repeated, the knife used being sterilized in the flame of a Bunsen burner between each cut. When the square was so small that high powers revealed nothing within it but the required coccus, the gelatine external to the square was removed very cautiously with a hot knife, leaving the coccus isolated on a minute island of

gelatine. A sterilized flask containing nutrient liquid was then brought close to the slide, the portion of gelatine containing the coccus was transferred to it on the point of the freshly sterilized knife; the flask was closed instantly with sterilized cotton-wool, and placed in the incubator.

The flask used contained a solution of 5% cane sugar, with a trace of black sugar, which had been boiled, steam sterilized, and twice bacterially filtered, and had been incubated at 35° C. for six weeks without showing a sign of any growth; hence it was incontestably sterile previous to inoculation.

Professor Marshall Ward [38] has laid down the dictum that the only method of diagnosis between Schizomycetes and Hyphomycetes is that of the hanging drop; he does not seem to have contemplated the possibility of a case in which this method by the nature of things is not applicable. When, as in the present instance, such a case does arise, then either the organism must remain unclassified as not electing to conform to the regulations of Professor Marshall Ward, or the proposed method of diagnosis cannot be accepted as final. In company with such observers as Zopf and Migula we prefer to adopt the latter course.

Stains used, and Method of making Permanent Preparations.

Fifty-four permanent microscopical preparations were made not only of the organism as in rum, but as cultivated in various stages. The stains used were as follows:—

I. Dahlia.
II. Methyl-green.
III. Safranin.
IV. Eosin.
V. Haidenhain's iron-haematoxylin.
VI. Gram's stain (iodine with and without gentian violet).
VII. Aqueous corallin.
VIII. Iodine green.
IX. Aniline green.
X. Gentian violet.
XI. Orange G.
XII. Fuchsin.
XIII. Fuchsin S.
XIV. Biondi-Haidenhain's triple stain.
XV. Ziehl-Neelsen's carbol-fuchsin.

All these stains, and other reagents used, were obtained for

the purpose of this investigation directly from the well-known firm of Grübler & Co., Leipzig.

The method pursued in making preparations was as follows:—The usual rather rough-and-ready bacteriological method of fixing by heat was entirely avoided, as liable to cause distortion and artefact appearances. In the case of preparations made from rum direct, a drop of the spirit containing numbers of cocci was added to a drop of Hermann's fluid on a slide, and allowed to evaporate in the cold; it was found necessary to smear previously with vaseline all parts of the slide except that to be covered by the drop, as otherwise the strong spirit in evaporating would rush all over the slide, and the cocci would thus be carried away to the edges, instead of remaining *in situ*. The cocci gradually sank to the bottom of the drop, and were not only physiologically 'fixed' by the Hermann's fluid, but also caused to adhere closely to the slide. When the drop had evaporated nearly to dryness, and the crystals were beginning to form round the edge, every trace of vaseline was removed, and the slide washed thoroughly with distilled water to get rid of the crystals, the sugar, and as much of the débris as possible. The slide was then placed in stain in a stoppered tube, containing enough of the reagent to cover three-fourths of the slide, and large enough to hold two slides placed back to back; the slide was then thoroughly washed again with distilled water. As the use of alcohol over 30% strength was found to decolourize instantly, dehydrating was effected by drying very completely in the cold. The preparation was then treated with xylol and mounted in a solution of Canada balsam in xylol.

In the case of preparations from culture fluids, the same process was used with the omission of the vaseline, and more care taken to remove all traces of sugar by washing.

CHAPTER VI

LIFE-HISTORY OF THE MICRO-ORGANISM AS DEDUCED FROM CULTURES.

(*a*) *The Cocci as living in Spirit.*

THE cocci occurred most frequently in pairs; even in the large fused groups and in chains two individuals were generally seen to be more closely connected. Single cocci were, however, also abundant, each surrounded by a thick gelatinous envelope; they were round in shape and appeared perfectly homogenous. They varied considerably in size, ranging from 1 μ to 5 μ in diameter; the gelatinous envelope averaged $\frac{3}{4}$ μ extra in thickness. In coloured rum the cocci were always yellowish-brown, but, from our observations on the cultivated form, we considered the tint of the spirit-living cocci to be due to their taking up caramel from the colouring matter. This view was confirmed by the fact that in uncoloured spirit the cocci were all colourless, while a sample of the colouring matter used was found to contain the brown form only.

The refractive envelope surrounding the cocci, whether singly or in groups, only attained great thickness in spirit, and reminded one strongly of the similar envelope of *Leuconostoc mesenteroides*, a bacterium described by Van Tieghem and Cienkowski [6] as occurring in the beet-sugar factories of Java.

The fact that the only reagent which would stain the envelope was the same in both cases, viz. corallin, rendered the resemblance more striking, and at first led us to infer a relationship, especially as the optimum temperature for the culture of both was the rather unusually high one of 35° C.

Another point noticeable about the cocci in spirit (and in certain cultures) was that large groups were often seen to be surrounded by a yellowish cloud of slime (Pl. I. Fig. III). This cloud was not resolvable into elements by a magnification of 1,200 diameters, but from observations on cultures in which the same thing occurred, we came to the conclusion that the slimy cloud always contained, though it did not consist of, cocci of infinitely small but variable sizes, differing only from the ordinary kind in size and in their degree of affinity for stains. The most minute hardly stained at all, those slightly larger were less and less resistent as they approached the full size.

Whether these minute cocci are to be regarded as spores is a point to be considered hereafter.

Reproduction by transverse division in equal halves in a plane at right angles to the direction of growth was the sole method observed in the cocci while living in spirit; this frequently occurred with such rapidity that chains were formed, also large groups, the units composing which were so imperfectly separated from one another as to present an appearance not unlike drops of some oily substance partially run together (Pl. I. Fig. I, *a*, *b*). Division took place by a simultaneous constriction of the gelatinous envelope and the enclosed coccus. It is probable that this method of reproduction was the only one practicable during life in spirit, on account of the necessity for retaining a protective envelope of the necessary thickness.

(*b*) *The Cocci as cultivated in Liquid Media.*

The cocci as cultivated lost their brown colour in the course of a few days; they were then colourless and refractive, and measured from $1\frac{1}{2}$ μ to $4\frac{1}{2}$ μ in diameter; in form they varied from perfectly round to oval; some were slightly pyriform. No structure definitely distinguishable as a nucleus could be detected; some of the cocci appeared homogeneous, but more generally they contained granules which, from their staining reactions, were considered to be chromatin. The gelatinous envelope was present in those grown in liquid media, but was thinner and difficult to see; cocci cultivated

in gelatine (deep cultures) were entirely without the envelope. These last-mentioned cocci were always surrounded by a yellowish cloud, which was very slimy in consistency, and exactly like that described above as sometimes surrounding the cocci in rum.

Our culture media and methods of inoculation have been fully described above; the following is an historical account of the results obtained. For inoculation purposes we always used the cocci (1) from undiluted rum, (2) from cultures, and from both obtained the same results, namely, the production firstly of a rod-like motile form, and secondly of a filamentous growth, exhibiting true dichotomous branching, bearing cocci which were subsequently constricted off and were apparently capable of reproducing the same cycle. The controversial questions entailed by the record of such a life-history are of great scientific interest, and this part of the subject will be found fully discussed in succeeding chapters. At present it is our intention to confine ourselves to an exact relation of the facts observed by us.

The rate of growth was remarkably slow; at least six weeks, and under unfavourable conditions a longer time, was required to complete the cycle of development from coccus to coccus. The sequence of events observed was as follows:—in flask cultures in liquid media, inoculated with more than one cell, the cocci increased in number by transverse division in equal halves in a plane at right angles to the direction of growth, until a thick sediment, amounting in some cases to heaped colonies, was formed. After a few days the liquid, originally quite clear, presented a turbid appearance, accompanied, in the case of coloured sugar solutions, by strong fluorescence resembling that of faulty rum; by the sixth day the culture was swarming with actively motile rods, as figured in Plate III. Fig. XIV. The length of the rods varied from 5–9 μ, single segments averaging $2\frac{1}{2}\mu$ and their thickness nearly 1 μ; they had a peculiar and characteristic appearance due to differentiation into highly and less refractive portions, and it is notable that in this respect they corresponded in appearance and staining reactions both with the cocci and the filaments.

At first we considered these rods to be an impurity, and threw away every culture which contained them; we were, however, struck by the fact (1) that they persistently occurred in every bacterially filtered liquid medium used except milk sugar, (2) that they were developed in liquids inoculated both from previous cultures and from rum direct, (3) they appeared in diluted uncoloured rum some weeks after dilution, though not in the boiled water used for dilution nor in the undiluted spirit, but (4) never in blank controls. In consequence of these observations, instead of throwing away the cultures which developed rods, we put them aside in the incubator, apart from the others, and allowed the development to take its course.

Observation then showed that, in the course of a few days, during which the rods increased both in length and thickness and resembled single floating filaments, sometimes branched, they became entangled in groups, which at first remained suspended in the liquid, but ultimately, as they became larger and heavier, sank to the bottom of the flask. When this took place, the liquid became clear.

The filaments, matted together, grew radially, thus giving rise to the ball-like growth seen in Plate IV. Fig. xv. In every case such coccus-bearing filaments were ultimately developed, and were identical with those grown in milk sugar, in which medium no rods occurred. The inference, therefore, seemed unavoidable, that we were dealing not with an impurity, but a stage in the life-history of the micro-organism of faulty rum; but this inference was set aside until a successful method of single-cell culture could be devised, or until we could prove by direct microscopical observation of a single individual that the rod-like form belonged to the normal cycle. At the earlier stage of the investigations, owing to the special difficulties described above, we had not been successful with single-cell methods, and these difficulties were further increased by the fact that we were dealing with a motile form; our ultimate success will be described under the head of single-cell cultures.

In the case of milk-sugar cultures, in which no rods appeared, the cocci increased in number by division until they formed a thick sediment and eventually round heaped colonies.

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Under favourable conditions, viz. in not too concentrated solutions, these heaped colonies developed filaments, which grew very slowly, ultimately attaining a length of nearly half an inch. The earliest period at which filaments began to develop was ten days after inoculation; they were first observed in cultures which had been kept under glass in direct sunlight, and consequently at a varying temperature. In the case of cultures kept in the dark at a constant temperature, the filamentous growth generally developed later and more sparingly. In certain of the cultures kept under glass and exposed to sunlight, but inoculated with a double dose of the coccus-containing liquid, the filamentous stage was entirely suppressed, though the stage immediately preceding it (marked by the formation of heaped colonies) was reached. After seven weeks' growth sufficient nutrient medium was added to bring these cultures up to the same proportion as those which had received the single dose at inoculation, and in all cases a growth of filaments then took place immediately, and the normal cycle was completed. The filaments almost always remained submerged, and generally anchored to the parent heap, but portions artificially detached continued to grow radially while floating freely in the nutrient medium. In one culture only (in a 5% solution of milk sugar) the cocci, instead of becoming heaped at the bottom, formed a scum on the surface, and in this case the filaments grew downwards into the liquid, and the exposed surface of the colony assumed a smooth waxy appearance.

The filaments, as seen when removed from the nutrient medium into distilled water, were of the purest white when fresh; old cultures sometimes acquired a blackish-green tinge. They exhibited true dichotomous branching (Pl. II. Fig. v), possessed a distinct double-contoured sheath, and were divided by transverse septa into segments of varying length, which contained or bore cocci. These septa were straight in young branches, but in older filaments showed a tendency to become concave, the concave surface being invariably directed towards the terminal end of the filament. The contour of a full-grown colony was almost globular, the filaments radiating towards

the periphery from an apparently denser central mass, and presenting somewhat the appearance of a ball of swansdown. The filaments were slimy to the touch, and interlaced closely and tenaciously, but never anastomosed, so that they could hardly be said to form a true mycelium. Nor was there any real morphological difference between those filaments which bore cocci and some which did not, though the goblet-like swelling of some filaments, when cocci were being constricted off from their tips, gave rise to an appearance resembling the conidiophores in certain moulds. The cocci did not lie loose within the filaments, for pressure on the cover-glass did not cause them to move along, nor could they have been extruded at the tip, as in Crenothrix, since the septa would stop the egress of all but the cocci in the terminal segment. The substance of the filament was perfectly continuous with the envelope of the cocci, as was plainly evident where the latter were being constricted off. This process took place at any point in the length of the filament as well as at the tip (Pl. II. Figs. VIII, IX, XII), and varying numbers of cocci, from single individuals to chains of six or more, were nipped off at a time from one point.

The cocci thus thrown off were very large, had very thick envelopes derived from the substance of the filament, and generally contained from one to five minute colourless roundish bodies, which were often extruded afterwards by the bursting of the envelope surrounding the parent-coccus (Pl. II. Fig. IX, *c*). These bodies were homogeneous and highly refractive in a fresh preparation, but stained very deeply and then appeared granular. The filaments were tapering in form towards the tip, and extremely variable in thickness, viz. from 1 to 3 μ in different branches of the same growth. The youngest colony removable on the point of a needle is figured on Plate VII Fig. XX.

Of liquid media other than milk sugar, the solution of coarse black sugar, boiled, steam sterilized, filtered once through paper, and twice through a Müncke's filter, was found to be unquestionably the best, and our most successful cultures were made in the 5% solution.

The filaments were abundantly developed in this, markedly the best in flasks incubated in the light at 35° C., next best in those exposed to direct sunlight under glass at a varying temperature, while in flasks incubated at 35° C. in the dark there was very late development of filaments and much slower growth.

In every culture without exception, and consequently in every medium used, as well as from inoculations of the most varied character possible, the sequence of events was as described, and filaments were developed.

The Cocci as cultivated on Solid Media.

The following results were obtained in deep cultures in test tubes inoculated as described on p. 29.

Growth was marked by the appearance of a slight cloud, which became more and more defined, and consisted of cocci surrounded by a yellowish slime, so glutinous in consistency that it was difficult to remove portions for examination without extracting the whole mass. The gelatine without sugar gave, contrary to our expectation, the best growth, but the cocci were devoid of any envelope, yellowish in colour, and very small, and no filaments were ever developed.

Transplanted with a very thin layer of gelatine on to microscope slides, growth took place freely, and the colonies formed presented very curious and interesting features. When thus growing on the gelatine without a cover-glass, the young colonies exhibited a raised corrugated surface, water-grey in colour, with elevations of a most peculiar crater-like shape (Pl. III. Fig. XIII), which we believe to be characteristic of no other micro-organism hitherto described. The crater-shaped central hollows afterwards tended to fill up, so that older colonies assumed a more rounded contour, the corrugations of the surface gave a convoluted or arborescent appearance (ibid.). The whole formation was perfectly hard and glassy ; if scratched with a sharp-pointed knife, both the sound and the scratch were like those made by a skate on ice. The colonies first became visible as minute rosette-like specks, with a central 'crater-' like hollow ; always surrounding the periphery

was a fringe of highly refractive processes with peculiar three-knobbed growing-tips (Pl. III. Fig. XIII, *a*, *b*); these knobs later grew out as branches. As this fringe grew from underneath the colony, and the latter was opaque, it was not possible to determine the earliest origin of these processes, but they were considered as corresponding to the filamentous stage in sugar. Nor was it easy to determine with exactness the mode of formation of the 'crater,' because the layer of gelatine, though thin, was semi-opaque, and the cocci were embedded in it; also because, from the nature of the colony, a cover-glass (and consequently an oil-immersion objective) could not be applied. It appeared, however, from observation with the highest powers possible under the circumstances (800 diameters), that the 'craters' were formed by the juxtaposition of cocci, cemented together by a slimy secretion, which hardened into the glassy ice-like substance above described. Probably the latter substance and the slime previously observed were identical in nature, and should be considered as different states of the same secretion. Slide-cultures made under cover-glasses yielded exactly the same results as test-tube cultures, but where some of the cocci accidentally extended beyond the edge of an unsealed cover-glass, crater colonies were again formed. With the view of investigating this formation more closely, a great number of cover-glasses were spread with the thinnest possible layer of gelatine by pouring the fluid gelatine on them while hot, draining off again, and then allowing the remaining gelatine to set, and inoculating with cocci from various rums. Some of these cover-glasses were then placed, gelatine side downwards, over glass cells such as were used for moist chambers, so that the development of the craters might be observed from the under side, with the oil immersion. As the gelatine was in contact with the air in the cell craters were formed, and our observations showed that they arose by the hardening of the slime secreted by, and immediately surrounding, each coccus. For some reason the coccus was not at first covered completely by the slime, hence the formation of the crater-like central hollows in young growths, which corresponded to the position of the coccus. The slime, how-

ever, hardened more quickly, and consequently more craters were formed on cover-glasses, which were kept surface-upwards under a large glass vessel, where they had access to more air.

Knobbed processes were not observed under these conditions, perhaps owing to the very small amount of nutrient medium, which was insufficient for their development.

Surface-cultures were tried on gypsum blocks moistened with (1) 5% solution of black sugar, (2) 5% solution of cane sugar with a trace of microcosmic salt, and (3) 5% solution of cane sugar without salts. These were inoculated, as in other experiments, both from rum direct and from previous cultures, but in no case was any growth obtained.

Culture for the Observation of Single Cells.

I. On Solid Media.

For shake-cultures in Petri's capsules a measured quantity of the infected rum was shaken up in a small quantity of liquid gelatine, which was then allowed to solidify in a very thin layer. Various brands of rum, both white and coloured, were used for inoculation. From all we obtained a growth of filaments identical with those grown in black sugar; and were for the first time able to trace under the microscope the development of these from a single cell (Pl. I. Fig. IV), and thus to complete the life-history from coccus to coccus.

The shaking after inoculation was done very thoroughly, in order to separate the cocci as much as possible. After the gelatine had set, the Petri-dish was placed under the microscope and searched for cells which were sufficiently isolated for each to develop without interfering with others. When a cell thus isolated was found, its position was marked by a circle drawn round it with a needle on the surface of the gelatine, and it was kept under direct observation under the microscope.

The mode of development was as follows: the coccus became of a long oval in shape, then from one end (or sometimes both) a tube-like filament was developed, in which septa were visible as transverse lines (Plate I. Fig. IV, *a*, *c*); when the filament

was about three segments long, it began to branch by dichotomous division of the terminal segment (Plate I. Fig. IV, *c*), the branches divided again, and more were developed by outgrowths from some one or other of the basal segments of the main stem. The order of succession was thus quite irregular, and there was nothing like acropetal growth. The development was the same throughout as that in milk sugar. The filaments, by interlacing closely, soon formed a dense white circular mass on the surface of and embedded in the gelatine (Pl. VII. Fig. XXI). Old cultures tended to become a blackish-green, and in every case the colonies produced a very strong and disagreeable smell, resembling that of valeric acid, a property not observed in any previous cultures.

II. In Liquid Media.

A. Transplanted to Flasks.

The method by which single cells were isolated on gelatine and transferred to flasks containing various liquid media has been described in Chapter V. The results obtained with these cultures agreed in every respect with those obtained in flasks containing similar media inoculated with more than one cell, and thus afforded tolerably satisfactory evidence of the purity of our earlier cultures. In every case the sequence of events observed was the same as in the earlier liquid cultures, other than milk sugar; the liquid became turbid, and by the sixth day contained numbers of the characteristic motile rods, which became entangled in groups, sank to the bottom, and gave rise to balls of filaments identical with those previously observed. Preparations were made both of rods and filaments from single-cell cultures, and stained with the same reagents. Other flasks were inoculated from the produce of the single cells.

B. Sown in Drops.

In order to obtain, if possible, direct proof that (1) the individual cocci developed into rods, and (2) the rods into filaments, the following series of cultures were made and kept

under direct observation with the microscope. Slides with a large central cavity were used, and the hollow of each was filled with the nutrient medium, and inoculated with as few as possible of the cocci, obtained by the dilution method, and either introduced directly into the drop or dried on the cover-glass. The drop was covered with a cover-glass ruled in squares, and the squares in some cases were further subdivided by marking with paint, in order to facilitate orientation. The cover-glass was carefully sealed with soluble glass on to the slide to prevent currents of evaporation, and the slide then examined under the microscope for isolated cocci. One was selected and its position noted by the squares on the glass; the slide was then clipped firmly in position and kept under observation for several days consecutively, the whole microscope, while not in use, being covered with a glass shade to prevent disturbance. The result of this experiment was that the coccus remained *in statu quo* when the last observation was taken at night, but by the next morning it was not visible in the field of view. Repeated trials always led to the same result. *Yet the coccus is not motile.*

As we desired to avoid all possible source of error, a slightly different method was adopted. A plain cover-glass was used, not ruled in squares, and the position of the coccus at the beginning of the experiment was noted by means of a Leitz camera lucida. The advantage of this particular camera for the purpose was its consolidation with the eye-piece, and as both together could be screwed into place, there was no possibility of moving after adjustment; further, its use obviated the necessity of a sloping board for the drawing paper, which could be pinned flat upon the table and thus could not slip out of place.

When the hollowed slide had been clipped firmly in position the isolated coccus was drawn on a card pinned to the table, and a small circle drawn around it. If there were any movement or alteration in shape of the object drawn, obviously its contour then would no longer coincide with the outline drawn, and thus the slightest displacement would be visible. The whole apparatus was left in place for several days, and

as a result it was found that when the last observation was taken one night the coccus had become slightly elongated, and by the next morning it had disappeared from within the pencilled circle, and immediately outside the latter there appeared a small rod in active movement, which was not there at the outset of the observation. This experiment was repeated eight times with single cocci, and once with five cocci drawn in the field at once. On every occasion there was the same sequence of events, namely, the cocci became elongated, and after a certain number of hours no cocci were visible, but a corresponding number of motile rods were found close to the positions previously occupied by the cocci. The inference, therefore, that a transformation of cocci into rods took place, was very strong, but owing to the extremely slow development of the organism, the uninterrupted watch necessary for absolute proof of the transformation would have required a number of skilled observers taking turns for several hours apiece during several days. The camera method above described was also adopted for the investigation of the further question whether rods developed into filaments. For these experiments the cavities of the slides used were larger, as it was necessary to provide the rods with a large bulk of nutrient medium. It was thus observed that the rods increased in length and thickness, became entangled in groups, and sank to the bottom, where, owing to the depth of the hollow, the further development was beyond reach of observation with high powers. As far, however, as the sequence of events could be followed, it will be obvious that the results accorded with the earlier observations.

CHAPTER VII

BIOLOGICAL AND CHEMICAL CHARACTERISTICS

Conditions of Life.

I. With relation to Oxygen.

As this bacterium survives for a length of time at the bottom of vats or puncheons of spirits, it is clear that it must be potentially anaerobic, as the amount of oxygen dissolved in the spirit is quite insignificant; our experiments indicated that it is preferentially aerobic, as it developed rapidly upon the thin surface of gelatine on a microscope slide without cover-glass or in Petri-dishes, but did not advance beyond the coccus stage when immersed in deep layers of gelatine, or in undiluted rum.

II. With relation to Light.

It has already been observed above that direct sunlight, instead of being inimical, appears rather to favour the development of this bacterium; this is probably to be expected of an organism of tropical habitat. Further, it appeared that in the rod stage it was even heliotropic, though at present we are unable to decide whether this condition results from luminous or non-luminous rays. Some observers (Migula) [7] have arrived at the general conclusion that though sunlight prevents the development of strictly pathogenic bacteria, yet the ordinary species of saprophytes develop as readily in diffuse daylight as in darkness.

III. With relation to Alcohol.

In the previous chapters we have alluded to the fact at once novel and remarkable of the survival of life in alcoholic liquids containing upwards of 70% of spirit.

Strictly speaking, the organism survives not in the alcohol, but in its gelatinous envelope, thus living, as it were, in a state of siege in its own castle, through the walls of which it can obtain its necessary supplies of food in the form of sugar, while keeping out its enemy alcohol.

It has been known since the work of Dutrochet [8], in the year 1827, confirmed by Graham [9], that of the commoner carbon compounds alcohol possesses the least, but sugar the most, power of endosmosis or exosmosis through animal membranes. In this connexion the well-known observation of Sömmering [10] and others may be mentioned, viz. that aqueous alcohol contained in a bladder and hung up in a warm room loses water by osmosis and consequent evaporation, nearly absolute alcohol remaining behind; this method was even formerly applied commercially, and we believe it to be still in use in some hot climates. We are not prepared to state that the bacterium, even though protected, can survive indefinitely in spirit, but we are still obtaining successful cultures from faulty rum, which has been in our possession for eighteen months.

Two experiments were conducted to ascertain the proportion of alcohol fatal to the bacterium; saturated solutions of cane sugar in absolute and 90% alcohol respectively were prepared, and 20 c.c. of each solution were inoculated with ·25 c.c. of rum containing an abundance of cocci. After incubation for a few days the cocci were found to be dead, as they were shrivelled up, and no growth could be obtained when transferred to favourable media. It would thus appear that the limiting proportion of alcohol in which the organism will continue to survive even for a short time is intermediate between 75 and 90%, and this limit is confirmed by the experience of the manufacturers, who have found occasionally that if the spirit is brought over at a rather higher strength

the evil, arising from the reintroduction of the organism by the colouring matter or otherwise, is to a greater or less degree overcome.

Pathogenicity.

In order to determine if the bacterium possessed any pathogenic properties, small portions of two separate cultures in gelatine were rubbed up with sterilized distilled water; the extract was injected subcutaneously into a guinea-pig. Up to the present time, viz. some months after the experiment, no effect has resulted, as the animal has remained in a normal state of health throughout the whole of the period; it is, therefore, improbable that any effect would be produced upon the human organism.

We are indebted to the kindness of Dr. J. Ritchie for performing the above experiment for us.

Habitat.

Unfortunately we have been unable to obtain from the planters sufficiently definite information to enable us to trace out the original habitat of the micro-organism.

We consider it probable that it is not truly indigenous, but was accidentally imported; it has certainly found a nidus in the sugarcane extracts, and is reintroduced after distillation with the caramel used for colouring or from infected vessels. Possibly it may occur in the water supply, but in our opinion the latter is not the most probable source. We have found that the cocci may be desiccated and air-borne, as our great difficulty, at the time when samples were being examined and cultures made, was to keep out the organism from any vessel in the laboratory, which was exposed to air.

The fact that some cocci, which had dropped off microscopical preparations while these were being placed in the staining-tubes, gave rise to vigorous growths in several aqueous stains, showed that the organism possessed extraordinary powers of resistance, even after having been dried and treated with Hermann's fluid.

Chemical Changes.

Production of an Acid.

In the course of the preceding chapters it has been shown, firstly, that the organism probably increases to a slight extent the proportion of acid in diluted faulty rum; and, secondly, that a sample of caramel containing the organism was acid, while another free from it was neutral.

Further, it was frequently observed in the course of the investigation that the various culture media, originally of neutral, became markedly of an acid reaction. In order to test this point weighed quantities of calcium carbonate were introduced into two (5%) solutions of sterilized cane sugar, one of which was inoculated with filaments and the other with cocci from previous cultures in raw sugar, both being kept in the incubator at a constant temperature of 35° C. for nineteen days. In both cases there was an abundance of growth, and a proportion of the calcium carbonate was found to be used up by neutralization, as shown by the following results:—

	Experiment I. Cocci.	*Experiment II. Filaments.*
Weight of calcium carbonate introduced .	·508 gram.	·502 gram.
„ „ recovered .	·465 „	·4559 „
Percentage „ neutralized .	8·47	9·19

The results obtained in the two experiments are fairly concordant, and though in the process of inoculation a trace of free acid was of necessity introduced, yet this may be considered as negligible.

A similar experiment was conducted on a larger scale, namely, with 10 grams of calcium carbonate and half a litre of a 5% sugar solution, with a view of isolating the acid or one of its salts; this was inoculated with cocci taken direct from the rum, and the whole mixture allowed to stand for five weeks. In this case there was a certain development on the side of the vessel nearest to, but comparatively little on the side removed from, the light, and the solution had become fluorescent. The mixture was then evaporated to dryness over the water-bath, and the residue frequently extracted

with hot water to dissolve out any calcium salt of an organic acid, and the solution subsequently evaporated, but it was not found possible to obtain a sufficient quantity of salt for identification.

The odour of the gelatine cultures in the Petri-dishes mentioned above tended to show that the acid produced was probably valeric, and not lactic, butyric, or acetic acids, which are more commonly formed.

Reduction of Nitrates.

In order to examine if the micro-organism possesses the property of reducing nitrates to nitrites or ammonia, the culture liquids were made up according to those used by P. F. Frankland in his investigations upon the subject [11]. It was proved that the liquids, previous to inoculation, were free from nitrites by the metaphenylene-diamine reaction, and from ammonia by Nessler's solution. After inoculation for forty days, and production of a vigorous growth, the liquids were again examined; no nitrites were found, but plenty of ammonia in the first portions which came over on distillation. As the quantity of ammonia was out of proportion to the peptone used, it is probable that its formation is due to the reduction of the nitrate by the organism, and not wholly to the decomposition of the peptone.

Hydrolysis of Starch.

Experiments made with this view led to negative results.

Production of Colouring Matter.

It has already been pointed out that the bacterium assimilates the fluorescent colouring matter from caramel and black sugar, but it appears that under certain circumstances it can of itself form a colouring matter. Thus all the growths in gelatine, even when sown with cocci from white rum, became yellow or reddish yellow with a distinct green fluorescence, these being the characteristics of Migula's [12] first group of colouring bacteria. In order to afford further confirmation,

a gelatine culture, sown with white rum, was extracted with water, when a yellow solution was obtained with a slight green fluorescence; the solution was divided into two equal portions, to one of which a solution of potash was added, which produced a deep moss-green colour, while the other portion was diluted with an equal bulk of water by way of comparison (Thumm's [13] reaction). Zopf's [14] lypocyanine reaction with concentrated sulphuric acid gave a negative result, thus showing that the colouring matter was not a lipochrome, hence the organism did not belong to Migula's second group. In another old culture containing peptone a bright yellow colouring matter was found which turned a deep red on addition of alkali; it is merely desired to place this last observation on record without however wishing to lay stress upon it.

Staining Reactions.

The cocci in coloured rum stained well with gentian violet, and with Gram's method, but, owing to the deep yellow colour already present, only such very powerful stains were at all effectual on these; the colourless cocci from uncoloured rum stained more intensely with Gram's method. The bacteria parted with stains, however, as readily as they took them up; treatment with clove oil or alcohol of over 30% strength decolourized them instantly. The only exceptions to this rule were preparations which had been purposely much overstained with gentian violet, and those treated with a strong mordant, as in Gram's method. The cultivated cocci stained readily with nearly all the stains mentioned on p. 32, except with eosin, fuchsin, and fuchsin S, which were practically found to be useless, however long applied. The best results were obtained with a double stain of gentian violet and orange G, and with Haidenhain's iron-haematoxylin; the gelatinous envelope of the cocci remained unstained, except with aqueous corallin. It was not found possible to obtain a contrast stain for the cocci, on account of the readiness with which the corallin was extracted by other reagents. Two points were noteworthy with regard to the behaviour of

the bacteria with reagents, viz. (1) preparations of cocci and filaments had the effect of precipitating many stains—especially eosin, iron-haematoxylin, and methyl-green—so rapidly that these could seldom be used a second time without filtering; and (2) the bacteria invariably stained a beautiful *blue* with iodine green and methyl-green, both of which should give a green stain; the blue appeared on the first washing with distilled water. The inner substance of the filaments stained lightly with most of the reagents used, but the sheath was quite unstainable except with Gram's strong iodine, and with aqueous corallin; the latter stained this structure only, leaving the contained cocci untouched. Gram's strong iodine stained the sheath of the filaments bright yellow, but subsequent treatment with sulphuric acid produced no blue colouration.

The cocci constricted off from the filaments, whether terminally or laterally, were identical in size and appearance with the original cocci, and stained in precisely the same manner with the same reagents. The small bodies extruded by these cocci stained intensely with almost all reagents used (Pl. II. Fig. IX, *d*) and then appeared granular. Cocci which had extruded such bodies stained much paler than those which had not (ibid., *a*, *b*).

This affinity of the small granular bodies for stains indicated that they were not of the nature of spores.

The rod or bacillar forms stained intensely violet with Gram's method, and also with Ziehl-Neelsen's carbol-fuchsin, applied hot. With the former the stain was uniform, and too heavy to show any structural details, but with carbol-fuchsin the rods showed very well their characteristic division into highly refractive and less refractive portions, the latter staining purplish brown, while the former remained unstained (see Pl. III. Fig. XIV). Iron-haematoxylin showed a similar differentiation. Löffler's method for staining flagella was also used for the rods, but though the modifications of this method suggested by Winogradsky [15] and Moore [16] were both tried, we did not succeed in showing the presence of flagella.

CHAPTER VIII

DISCUSSION OF CONCLUSIONS

It will be seen from the preceding chapters that if the life-history of the micro-organism is as described by us, it presents points of resemblance to various groups, and that consequently the determination of its true systematic position is very difficult. The question depends wholly upon the purity of our cultures, and we have described in the fullest detail every experiment performed and every method used, in order that those of our readers, who are bacteriologists with practical experience, may be enabled not only to judge whether these methods were reliable but also to repeat them, if so desired.

Meanwhile it is clear that, with regard to the results of our investigation, three views could be held legitimately.

(1) The cocci are the conidia of a mycelium-forming hyphomycete (mould) and the rods are bacterial impurities.

(2) The cocci are bacterial in nature and become rods as described by us, but the filaments belong to a hyphomycete.

(3) The cocci, the rods, and the filaments are phases in the life-history of a single organism belonging to the higher bacteria, and probably allied to the Streptothrix group.

The arguments for and against each of these views will be fully discussed seriatim, though, owing to the special difficulties often alluded to above, positive proof has not here been brought forward.

First View.

In favour of this it may be said that (1) the cocci as in rum are variable in size, and generally large as compared with the majority of bacterial cocci, (2) they are able to reproduce the cycle without the interpolation of spores, (3) there is the

direct microscopical evidence of their continuity in development with the filaments, (4) there is also the possibility (always present) of the introduction of the spores of a foreign bacillar organism either at the moment of inoculation or in the inoculating substance itself.

Against these points the following arguments may be advanced: (1) the cocci, though large for bacteria, are certainly somewhat small for conidia; (2) the character of their division; (3) the ability to reproduce the cycle directly from conidia-like bodies is equally characteristic of the higher bacteria of the Streptothrix group, and the strong inference from facts observed that the cocci as in rum become transformed into rods; (4) the difficulty of imagining the rods to be impurities, considering the circumstances under which they occur, the precautions taken, as well as the absence of any other direct evidence of the existence of a second organism, unless as invisible spores, in the undiluted spirit.

Second View.

The arguments in favour of this may be summarized as follows—(1) The reproduction of the cocci by transverse fission, the products being at the time of division of equal size, this mode being characteristic of the Schizomycetes (bacteria); (2) their staining reactions in which they resemble bacteria; (3) the observations above alluded to, showing that the probability of the transformation of cocci into rods almost amounted to a certainty.

This point has further been confirmed by Professor Emil. Chr. Hansen, who very kindly made a few experiments with some of our materials (namely, coloured and uncoloured undiluted faulty rum and some of our cultures) with which we presented him. He wrote to us as follows—'Your observation of the coccus becoming elongate and going into the form of rods is probably quite correct,' and added that in the few trials he was able to make with our materials, the 'rods and the coccus developed only bacteria.' Professor Hansen did not, however, agree with our conclusions as to the filaments, and

as his opinion is the only strong evidence we have in favour of the second view, and against the genetic connexion of these filaments with the rods and cocci, we think it best to quote his experiments as they were made known to us in correspondence. After having invited our attention to the fact that the filamentous growth 'might represent a phase in the development of a Penicillium species,' he wrote to us later, 'An experiment with filaments from the cultures you left here (cultivation on wort gelatine 25° C.) showed that the filaments really formed a Penicillium form.' In a second letter he wrote that in his experiments 'the hyphomycetous fungus (the mycelium form) only developed this form but not bacteria.'

On the other hand, he was willing to admit that under the circumstances the problem, whether one or two organisms were present, was very difficult, would require a long time, and might even prove insoluble, as far as attaining positive evidence was concerned.

The Third View.

The idea of a genetic connexion between the bacteria and moulds (Schizomycetes and Hyphomycetes) is, of course, not novel, but has been advanced at different times by several investigators. It must be admitted that hitherto, when subjected to strict experimental proof, sufficient evidence has not been forthcoming. A work recently published by A. Klöcker and H. Schiönning [17] shows that the 'moderne Pleomorphie-Wut,' to use their not inapt phrase, has even resulted in assertions that certain moulds can produce both yeasts and bacteria, though the originator of these views failed to satisfy the legitimate inquiries into his materials and methods.

The question, however, of a possible *bacterial* link between truly branching filamentous forms such as Streptothrix (originally known as Actinomyces), now regarded by most writers as true bacteria, and the Hyphomycetes is, we venture to think, still an open one, and the study of new and aberrant forms, such as the organism now before us, may furnish

possibly some means for arriving at a decision. The resemblances between our organism and the group associated by Migula under the title of *Chlamydobacteriaceae*, in which are included Streptothrix and others, are in many respects most striking. The following passage in his work (loc. cit.) may not inaptly be quoted :—

'Bei der am höchsten entwickelten Gruppe der Bacterien, den Chlamydobacteriaceen, scheint eine Endosporenbildung vollkommen zu fehlen, dagegen kommt eine Form der Fortpflanzung vor, die zwar bei den einzelnen Gattungen sehr verschieden entwickelt ist, doch aber allgemein an die Abschnürung von Gonidien bei den Pilzen erinnert und offenbar physiologisch dieselbe Bedeutung hat. Als allgemeines Charakteristicum der Bakteriengonidien kann man ihre Ablösung von dem Mutterfaden zum Zweck der Vermehrung der Individuen ansehen; sie stellen keine Dauerformen dar, sondern wachsen auf demselben Nährboden, resp. in derselben Flüssigkeit, in der Regel schon nach kurzer Zeit zu neuen Zellfäden aus. Sie unterscheiden sich von den Endosporen sowohl dadurch, dass sie nicht im Innern von vegetiven Zellen gebildet werden, als auch dadurch, dass sie keinen mit Abstreifung oder überhaupt Abhebung einer distinkten Sporenmembran verbundenen Keimungsprozess durchmachen und wie erwähnt nicht als Dauerzellen zu betrachten sind.'

The production of slime, the occurrence of motile rods, and of true branching are further points of agreement. On the other hand, though there are great resemblances in our organism to certain known Hyphomycetes, and especially to that described years ago by Fresenius [20] under the name of 'Torula rufescens,' which would now be called a species of Penicillium, yet there are also marked points of difference, especially in the branching of the conidia-chains in Torula. As far as we could observe, neither budding nor conjugation, nor formation of zygospores occurred in our organism ; further, the mycelium is not divisible into sterile and fertile portions, nor can it be said to possess distinct organs of fructification, since the cocci are often produced laterally at any point as well as terminally upon the filaments. We are informed, however, by Professor Hansen that he has observed the latter

peculiarity in the case of true Hyphomycetes, when growing under abnormal conditions.

But the mode of branching is not in all respects similar to that of any Hyphomycete, a septum seldom being found at the actual point of junction of the branch with its stem, whether the branch arises by the forking of a single terminal cell or by an outgrowth from a proximal segment. Further, the filaments never anastomose, however much they may interlace; their growth is also distinctly not acropetal, and does not correspond to any one of the several types of acropetal growth figured by Zopf [18 and 38]. Marshall Ward [19] uses acropetal and non-acropetal growth respectively as an absolute distinction between Schizomycetes and Hyphomycetes; Professor Hansen, on the other hand, finds that certain Hyphomycetes do certainly, under abnormal conditions, present non-acropetal growth. The bright yellow coloration of the gelatine taking place in deep cultures in which the development never proceeded beyond the coccus stage, and also accompanying the formation of filaments as well as of rods, is a bacterial characteristic, and appears to us a further indication that the three forms are phases in the life of one organism.

The reactions with Gram's and Löffler's stains, as also with corallin, eosin, and carbol-fuchsin, are especially bacterial, and tend to show that the envelope of the coccus, the refractive portions of the rods, and the sheath of the filaments are identical in nature, while the staining reactions of the inner substance of all these also correspond; this would be the case if the inner and outer substances were respectively derived, as we believe, from the same sources. The youngest growth of filaments of which stained preparations were made corresponded in thickness with the largest rods. It appears to us noteworthy that the rods never appeared in our blank control-flasks; hence it would seem that if they were impurities, they entered our cultures in the inoculating substance, and not from the air, especially as during the investigation the study of all other micro-organisms of fermentation was completely set aside. But even supposing the rods were impurities, it is difficult to explain their invariable absence in

milk sugar, whether with or without nitrogeneous substances, as also in those of our gelatine cultures which received the same inoculating medium supposed to contain spores. Finally, it appears to us extremely difficult to avoid the conclusion that the rods and filaments are genetically connected, when on the one hand direct microscopical observation proved continuity between the coccus from undiluted rum and the filament, and on the other both our observations and those of Professor Hansen tend to prove that exactly identical cocci, even from the same sample of rum, produced rods in another medium.

If the steps from coccus to rod, coccus to filament, and filament to coccus are thus placed almost beyond doubt, then the missing link is the rod to filament stage; this is only represented by our preparations, a number of which show the rods to occasionally exhibit true branching (Pl. VI. Fig. XIX), when the conclusion that they are single floating filaments is almost forced upon the observer. It should also be mentioned that the appearance of preparations of the youngest and smallest filamentous colonies obtainable indicates that they are not, at any rate, radial growths from a common point of origin.

In conclusion, we have not been able to find any description in the literature of Schizomycetes or Hyphomycetes of any organism exactly resembling that discovered by us in faulty rum, hence we must consider it as new to science. If our view is correct, our organism is excluded by one or other of its described characteristics from any group in any classification except that of Migula's *Chlamydobacteriaceae*, and from the genera at present included in this, namely, Streptothrix, Cladothrix, Crenothrix, Phragmidiothrix, and Thiothrix.

As, therefore, the organism, or organisms, has, or have, a real existence, it, or they, obviously cannot go forth into the world without a name, hence we propose provisionally to call it *Coleothrix methystes*[1], though we repeat what we have said in our preface, that the life-history of the organism may remain for some time to come a subject of legitimate controversy.

[1] Κολεός, a sheath, μεθυστής, a drunkard. We are indebted to Mr. A. Sidgwick, M.A., of Corpus Christi College, for the ingenious suggestion of this name.

CHAPTER IX

SUMMARY OF RESULTS

It has been shown in the foregoing chapters that the 'faultiness' of rum, first noticed about sixteen years ago, which has caused a great pecuniary loss to the manufacturers, is due neither to any resinous or other matters extracted from the casks, nor to saline or other substances precipitated, but to the presence of a micro-organism, hitherto undiscovered, which we have named provisionally *Coleothrix methystes*.

This organism, in the coccus stage of its existence, produces in rum primarily a turbidity, but also an increased fluorescence, owing to the assimilation by the organism of the fluorescent colouring matter of the rum. These phenomena have been proved, by optical tests, to exist in undiluted faulty rum, but become more apparent after dilution, in consequence of alteration of the refractive index of the liquid. Hence 'faultiness' has been rendered apparent by the brokers' test of dilution in the proportion of $\frac{1}{3}$ spirit to $\frac{2}{3}$ water. It has further been shown that faulty rum can be made sound, and sound rum faulty, by the removal and the addition of the organism respectively. The organism has been successfully cultivated in various media, and considerable evidence has been brought forward in support of our belief that it possesses three forms, viz. coccus, rod, and filament. The methods of culture and experiment have been fully described, as we hope, in such a manner that subsequent observers may repeat our observations; to this end the life-history of the organism under various conditions has been fully detailed, and illustrated by drawings and photographs.

An account is also given of the biological and chemical characteristics, as also of the staining reactions of the organism. The three possible views which might result from our investigations are discussed seriatim, and reasons assigned for our own view that the cocci, rods, and filaments are phases in the life-history of a single organism, belonging to the higher bacteria, and probably allied to the Streptothrix group.

In conclusion, we claim to have established the new facts (1) that life can exist in alcohol of nearly 75% strength, and (2) that the organism discovered by us is the cause of 'faultiness' in rum. With regard to the minor question of whether the forms cultivated belong to one or more species, positive proof is not at present obtainable.

Again, we would record our thanks to colleagues and fellow-workers for their advice and assistance in this investigation, wherein nearly all the various branches of natural knowledge are involved.

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EXPLANATION OF PLATES

Plate I.

FIG.

I. Cocci as in rum, (*a*) chains, (*b*) group, (*c*) dead cocci as in rum after fining. Camera lucida, oc. 1. obj. 7. Leitz; tube not drawn out.

II. Cultivated cocci from sugar-solutions, stained methyl-green. × 1000. Cam. luc.

III. Cultivated cocci, fresh state (with surrounding slime), before loss of yellow colour. Cam. luc. oc. 1. Oil imm. $\frac{1}{12}$, finished oc. 5. Leitz; tube not drawn out.

IV. Cocci developing filaments, from gelatine single-cell cultures. Cam. luc. oc. 1. obj. 7. Leitz; tube not drawn out.

Plate II.

V. Outline drawing of filaments from sugar, fresh state, to show true branching. Cam. luc. oc. 1. Oil imm. $\frac{1}{12}$, Leitz; tube at 16, finished oc. 5.

VI. Portion of similar filament, fresh state. Magnification as V.

VII. Single coccus, pair, and chains newly constricted off, fresh state. Magnification as V.

VIII. Filament, fresh state, with cocci constricting off terminally and laterally. Magnification as V.

IX. Penicillium-like filament, with cocci constricting off (*a*, *c*, *d*); cocci extruding granules. Stained gentian violet and orange G. × 1000. Cam. luc.

X, XI. Other filaments, fresh state. As V.

XII. Typical filament, with cocci constricting off. Stained safranin. × 1000. Cam. luc.

Plate III.

XIII. Culture on gelatine plate, showing crater-formation. Cam. luc. oc. 1. obj. 5. Leitz; tube at 16, finished oc. 5.

XIV. Rods from single-cell culture in sugar. Stained Ziehl-Neelsen's carbol-fuchsin. × 1000. Cam. luc.

Plate IV.

FIG.

XV. Freudenreich-Hansen flask containing filamentous growth fully developed. Nat. size.

Plate V.

XVI. Cultivated cocci, stained methyl-green. × 500.

XVII. Rods from single-cell culture, stained Gram. × 500.

Plate VI.

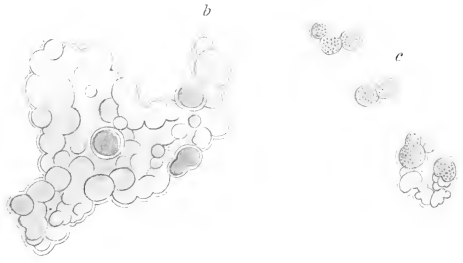
XVIII. Filaments with cocci. × 500.

XIX. Rods and developing cocci. × 500.

Plate VII.

XX. Very young filamentous growth from sugar. × 30.

XXI. Old filamentous growth on gelatine. Nat. size



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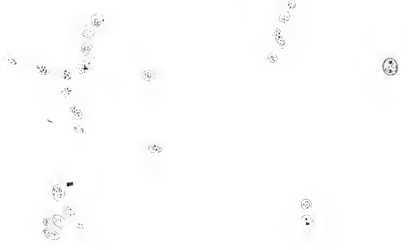

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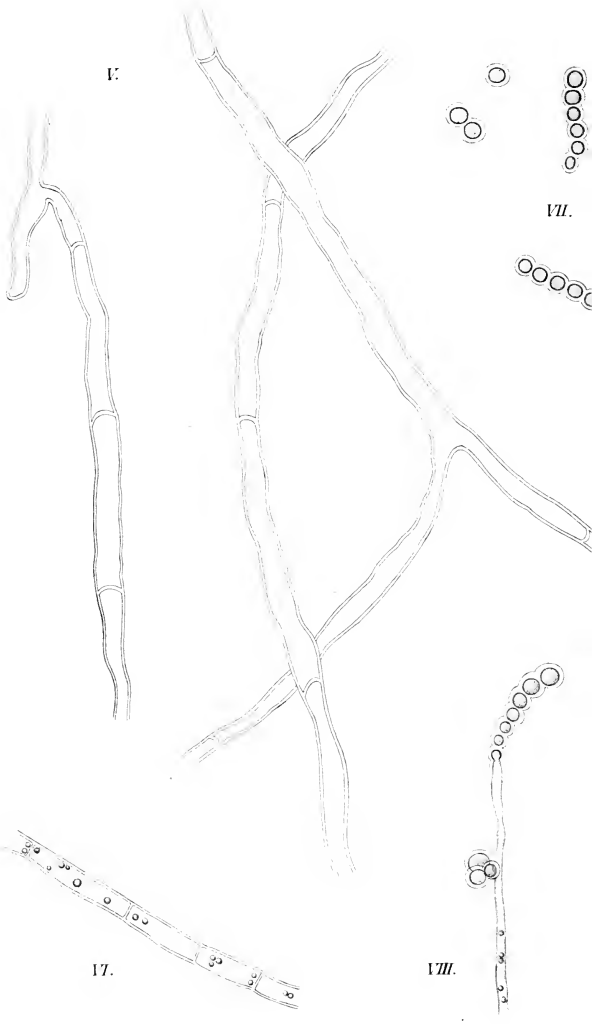

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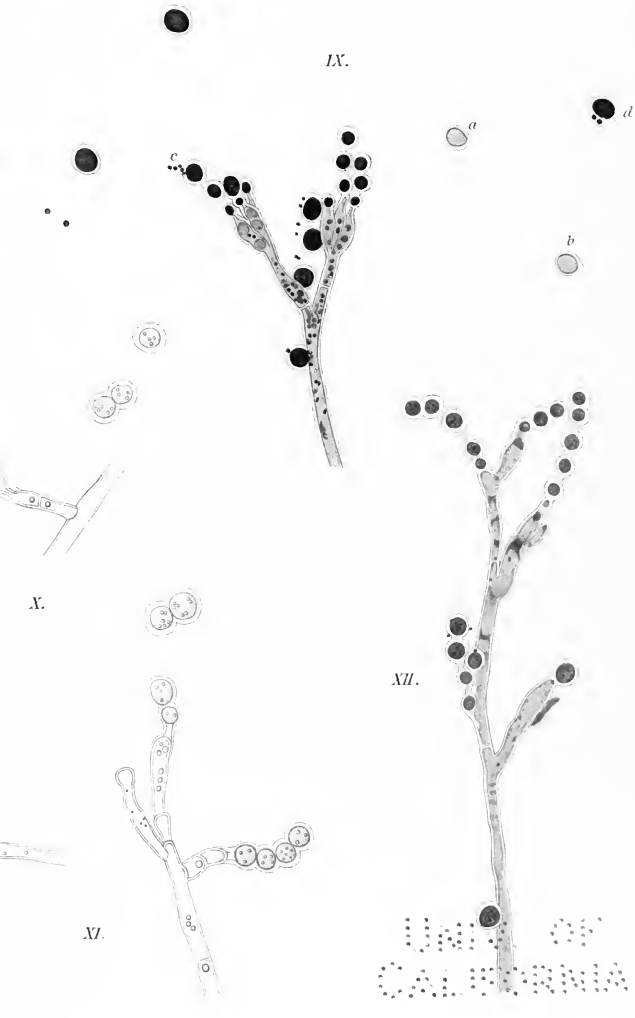

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Fig. XV

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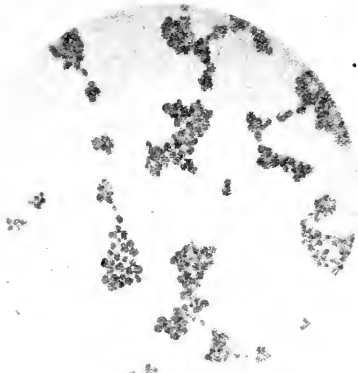

Fig. XVI.

Fig. XVII.

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